Tools and Technology Article

Validation of Fecal Glucocorticoid Metabolite Assays for South African Herbivores

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ABSTRACT Fecal glucocorticoid metabolite (FGM) assays are a popular means of monitoring adrenocortical activity (i.e., physiological stress response) in wildlife. Species-specific differences in glucocorticoid metabolism and excretion require assay validation, including both laboratory and biological components, before assay use in new species. We validated a commercially available radioimmunoassay (MP ^{125}I corticosterone RIA kit [MP Biomedicals, Solon, OH]) for measuring FGMs of several South African herbivores, including giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus buxtoni*), kudu (*Tragelaphus strepsiceros*), wildebeest (*Connochaetes taurinus*), and zebra (*Equus burchelli*). These herbivores are important in South African parks and reserves for ecotourism and as a prey base for predators and serve an integral role in ecosystem processes. Standard biochemical validations (e.g., recovery of exogenous corticosterone, intra- and interassay variation, and parallelism) demonstrated that the assay accurately and precisely measured FGMs of all 6 herbivore species. Our biological validations demonstrated that the assay was sensitive enough to detect changes in FGM production associated with season. Samples collected during the dry season (Jun–Aug) contained higher FGM concentrations than those from the wet season (Dec–Feb) in all species. We established optimal sample dilutions and reference FGM levels for these 6 herbivores, which can now be used to monitor the effects of management and ecotourism activities on the stress responses of these herbivores. (JOURNAL OF WILDLIFE MANAGEMENT 73(6):1014–1020; 2009)

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KEY WORDS adrenal activity, assay validation, fecal glucocorticoid assay, herbivore, physiological stress, radioimmunoassay, stress, South Africa.

In South African parks, officials make management decisions about wildlife based on the needs of local communities, interests of the park visitors, and many ecological considerations, including carrying capacity of the area. Managers attend to the interests of tourists by providing roads, lodging, and vehicles in natural habitat and by promoting accessibility for animal viewing. Increased vehicle and human activity can elicit a physiological stress response in wildlife (Wasser et al. 1997, Creel et al. 2002, Tempel and Gutiérrez 2004, Thiel et al. 2008), causing them to deviate from normal habitats, diets, and behaviors. Although response to an acute stressor can be adaptive in the short-term by redirecting resources to mobilize energy used, if conditions persist and the physiological stress response becomes chronic, the events can lead to decreased immune function, reduced reproductive capacity, and diminished survival (Sapolsky et al. 2000, Pride 2005). If ecotourism and wildlife health are incompatible, then wildlife conservation will suffer. The key to successful management of physiological stress in wildlife is accurate detection and monitoring of stress hormones (i.e., glucocorticoids).

Animals respond to external stimuli through multiple mechanisms and stress hormones are commonly used to measure adrenocortical activity. Stress hormones can be detected in blood, tissues, urine, and feces (Harvey et al. 1984, Wingfield et al. 1994, Wasser et al. 2000). However, with free-ranging animals the response to capture, handling, and blood collection might induce a physiological stress response and complicate data interpretation (Le Maho et al. 1992, Cook et al. 2000). Procedures have been developed to quantify adrenal activity noninvasively, through measurement of fecal glucocorticoid metabolite (FGM) levels (Graham and Brown 1996, Wasser et al. 2000, Millspaugh et al. 2002, Keay et al. 2006, Rothschild et al. 2008). In many studies investigating effects of multiple stressors, fecal studies have the added benefit of showing cumulative effects of stressors because they contain metabolites of stress hormones produced over a period, whereas plasma samples provide only hormone levels at one point in time (Harper and Austad 2000, Wasser et al. 2000, Millspaugh and Washburn 2004). After extensive hepatic and gastrointestinal metabolism (Bokkenheuser and Winter 1980, Schwarzenberger et al. 1991), there is little parent hormone remaining in fecal material and a variety of glucocorticoid

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metabolites are present (Goymann 2005, Palme et al. 2005). The assortment of individual metabolites can be different for each species (Goymann 2005, Mateo and Cavigelli 2005, Touma and Palme 2005). Because it is not native hormone being measured, it is essential to validate the assay to ensure that the species-specific suite of stress hormone metabolites is detectable.

Assay validation usually involves both laboratory and biological components. First, a standardized laboratory validation, involving parallelism and exogenous recovery tests, is performed (Jeffcoate 1981, Harper and Austad 2000). Second, it is necessary to determine whether the assay is sensitive enough to detect biologically significant changes in hormone excretion, using a biological validation (Washburn et al. 2002, Touma and Palme 2005). Biological validations can be accomplished pharmacologically by administering adrenocorticotropin to stimulate adrenal hormone production (Wasser et al. 2000) or by administering dexamethasone to suppress adrenal function (Palme et al. 2005). In wild animals, pharmacological manipulation is often not feasible and thus biological validation is accomplished by other means. For example, appropriate samples can be collected from groups of animals after exposure to stressful stimuli, such as capture or among animals during periods that are expected to increase stress hormone production (e.g., wet vs. dry season for African herbivores).

We assessed the feasibility of using a commercially available radioimmunoassay (RIA) to quantify FGMs as a proxy for physiological stress in multiple South African herbivore species through laboratory and biological validations. Our approach involved a biochemical validation in a field setting. We used wet and dry season contrasts to assess whether the RIA may be sufficiently sensitive to interpret what generally happens in the wild and whether optimal sample dilutions are appropriate under different conditions.

We considered a stressor to be any challenging or energetically demanding situation. We focused on several herbivores because of their importance as a prey base for predators (e.g., for reintroduced lions; *Panthera leo*), influence on ecosystem processes (e.g., nutrient cycling), and interest for ecotourism. Our objectives were to validate the RIA, determine optimal sample dilutions, and establish reference data for these herbivores.

STUDY AREA

We conducted our study in Tembe Elephant Park (30,013 ha) and Thanda Game Reserve (5,000 ha), South Africa. Both parks were located in northeastern KwaZulu-Natal, South Africa, approximately 100 km apart, in a geographic area known as Maputaland. The main tourist draws to both of these parks were the "Big Five" game species: elephant (*Loxodonta africana*), leopard (*Panthera pardus*), lion, white rhinoceros (*Ceratotheruim simum*), and buffalo (*Syncerus caffer*). Although these animals may be the main attraction, both parks sustained populations of many herbivore species that were integral to the ecosystem.

We collected fecal samples from May through August (dry season) and from December through February (wet season) during 2003–2005. The wet season has the greatest rainfall and supports greater plant growth, whereas the dry season has the lowest rainfall and less available forage. Mean annual rainfall in Thanda was 610 mm and 707 mm in Tembe (Matthews et al. 2001). The climate was warm to hot, with a mean minimum of 5° C and maximum of 38° C in Thanda and a mean minimum of 7° C and maximum of 35° C in Tembe (Matthews et al. 2001).

METHODS

We studied 6 species: giraffe (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus buxtoni*), kudu (*Tragelaphus strepsiceros*), wildebeest (*Connochaetes taurinus*), and zebra (*Equus burchelli*). These 6 species represent a wide taxonomic range, including both browsers (kudu, nyala, and giraffe), grazers (wildebeest and zebra), and mixed browsers and grazers (impala), accounting for several important large herbivores in the study areas. Some of these species share habitat and resources with rare herbivores, such as the endangered suni (*Neotragus moschatus kirchenpaueri*; Friedman and Daly 2004).

Field Procedures

We expected a priori that the dry season would result in higher FGM levels. We collected fecal samples at wildlife gathering areas, such as watering holes or clearings, where park employees frequently observed large groups of animals. We collected samples from males and females of all species during morning and evening hours from available watering holes and clearings, when wildlife tended to congregate. We attempted to sample wildlife gathering areas equally, but infrequent observations of some species (e.g., kudu) resulted in greater sampling at sites known to be frequented by these species. We attempted to observe animals without disruption by remaining in a vehicle on the road, away from the group, at a distance of 50–75 m. For all species, we observed animals defecating and collected samples once the group departed. Whenever possible, we collected samples from individuals that we witnessed defecating; when this was not possible we collected only fresh (i.e., moist) fecal pellets and avoided any large clusters of pellets that seemed to come from multiple individuals. We did not collect >1 sample per species at any one site to minimize likelihood of sampling the same individuals (i.e., pseudoreplication; Hurlbert 1984). We collected representative subsamples (i.e., multiple pellets constituting 50 g of material) from different parts of each fecal pile, to minimize variability of FGM levels within a pile or fecal bolus (Millspaugh and Washburn 2003). We did not collect pellets with urine contamination. We immediately froze samples and stored them at −20° C until processing.

Before shipment to the United States (in compliance with United States Department of Agriculture regulations for importation of ungulate fecal material), we submerged each sample in 2% acetic acid to minimize viable viral disease

particles, which has been shown to have minimal impact on hormone levels (Millspaugh et al. 2003). After 30 minutes, we drained the acetic acid from each sample vial and refroze the sample at −20° C. We shipped frozen samples to the University of Missouri, Columbia, Missouri, USA, for further processing.

Laboratory Procedures

We dried fecal samples in a lyophilizer for 24 hours. Once freeze-dried, we sifted samples through a stainless steel mesh to remove large particles; next, we thoroughly mixed each sample. We extracted glucocorticoid metabolites from feces by using a modification of Schwarzenberger et al. (1991). We placed 0.200–0.230 g of dried feces in a glass test tube with 2.0 mL of 90% methanol and vortexed the sample at high speed in a multitube vortexer for 30 minutes. We centrifuged samples at 2,000 × *g* for 20 minutes, removed the supernatant, and stored it at −84° C until assayed.

We used ^{125}I corticosterone RIA kits (MP Biomedicals, Solon, OH). We followed the MP protocol for the ^{125}I corticosterone RIA, except that we halved the volume of all reagents (Wasser et al. 2000).

For each species, we conducted standard assay validations, including parallelism and recovery of exogenous corticosterone tests (Jeffcoate 1981, Grotjan and Keel 1996, O'Fegan 2000) on 2 pooled samples during the wet season (e.g., expected lower FGM values) and 2 pooled samples from the dry season (e.g., expected higher FGM values); each pooled sample consisted of material from 3 individuals to confirm the assay was accurately and precisely measuring fecal glucocorticoid metabolites. Parallelism ensures the assay maintains linearity under dilution, and recovery of exogenous corticosterone verifies accurate measurement throughout the working range of the assay (Jeffcoate 1981). We used a high and low control (MP Biomedicals, Irvine, CA) to calculate interassay variation. We calculated intra-assay variation by averaging coefficients of variation of replicate tubes from 20 randomly chosen samples. We used data from 2 seasons to determine whether optimal sample dilutions are appropriate under different conditions.

To determine whether our assay was capable of detecting biologically significant changes in FGM production and excretion, we compared FGM levels of samples collected during wet and dry seasons. Although our study represents a biochemical validation in a field setting, the wet and dry season contrast helps determine whether the RIA may be sufficiently sensitive to interpret what generally happens in the wild. We chose sampling periods based on commonly accepted wet (Dec–Feb) and dry season periods (May–Aug). We selected ≥10 samples per species from both the wet and dry seasons. We extracted samples and ran them at the appropriate optimal sample dilution (Table 1).

We performed tests for equal slopes (parallelism) to determine whether log-transformed curves of serially diluted low and high pool extracts were parallel to log-transformed corticosterone standard curves (Neter et al. 1996). We considered exogenous corticosterone recovery of 90–110% acceptable (Jeffcoate 1981, Harper and Austad 2000, O'Fegan 2000) for validation of the assay. We used *t*-tests to compare wet and dry season sample sets for each species (Zar 1996). We considered test results significant at $P < 0.05$.

Table 1. Laboratory validation of fecal glucocorticoid metabolite assays in 6 South African herbivores. Average percent recovery of exogenous corticosterone ($n = 6$ per species; range of added corticosterone = 0.25–1.25 ng/mL) and optimal sample dilution factors for methanol extracts of fecal glucocorticoid metabolites. Optimal sample dilution provided the percent binding closest to 50% and thus is most likely appropriate for the full range of the assay. We collected samples from May through August (dry season), and December through February (wet season), during 2003–2005, in Tembe Elephant Park and Thanda Game Reserve, South Africa.

Species	Average % recovery	SD	Optimal sample dilution
Kudu	110.86	3.81	1:08
Nyala	108.99	1.68	1:32
Giraffe	109.76	3.42	1:16
Impala	109.71	4.57	1:08
Wildebeest	104.24	3.34	1:08
Zebra	109.60	3.32	1:04

RESULTS

For each of the 6 herbivore species, serial dilutions of low and high pool fecal extracts yielded displacement curves that were parallel to the standard corticosterone curve (all $P > 0.55$; Fig. 1A–F). Interassay variation for 4 assays was 7.6% and average intra-assay variation was 1.3%. We determined the dilution that provided a percent binding closest to 50% to be the optimal sample dilution for future assays (Table 1). Recovery of exogenous corticosterone was within the acceptable range (90–110%) for all species, except for kudu, which was 110.9% (Table 1).

In all species, average dry season sample FGM levels were higher than wet season levels (Fig. 2). Seasonal differences were significant for giraffe ($t = -3.49$, df = 31, $P < 0.001$), kudu ($t = -2.28$, df = 21, $P = 0.02$), nyala ($t = -3.94$, df = 25, $P < 0.001$), zebra ($t = -2.19$, df = 19, $P = 0.02$), impala ($t = -2.58$, df = 23, $P = 0.008$), and wildebeest ($t = -2.24$, df = 36, $P = 0.02$). There was great variability in FGM levels in all seasons for all species (Fig. 2). Fecal glucocorticoid metabolite levels for grazers were typically half, or less, than FGM levels for browsers. Percent increase between wet and dry seasons was 28% for grazers and 38.2% for browsers (which included impala).

DISCUSSION

Our findings suggested the MP Biomedicals ^{125}I corticosterone radioimmunoassay was capable of adequately measuring FGMs of 6 South African herbivores, which supports other research that has demonstrated effectiveness of this assay. Performance characteristics (i.e., recovery of exogenous corticosterone, intra- and interassay variation, and parallelism) of this assay verified that it was accurate, precise, demonstrated linearity under dilution, and had an appropriate range of sensitivity. The assay was sensitive enough to detect potentially significant biological changes in

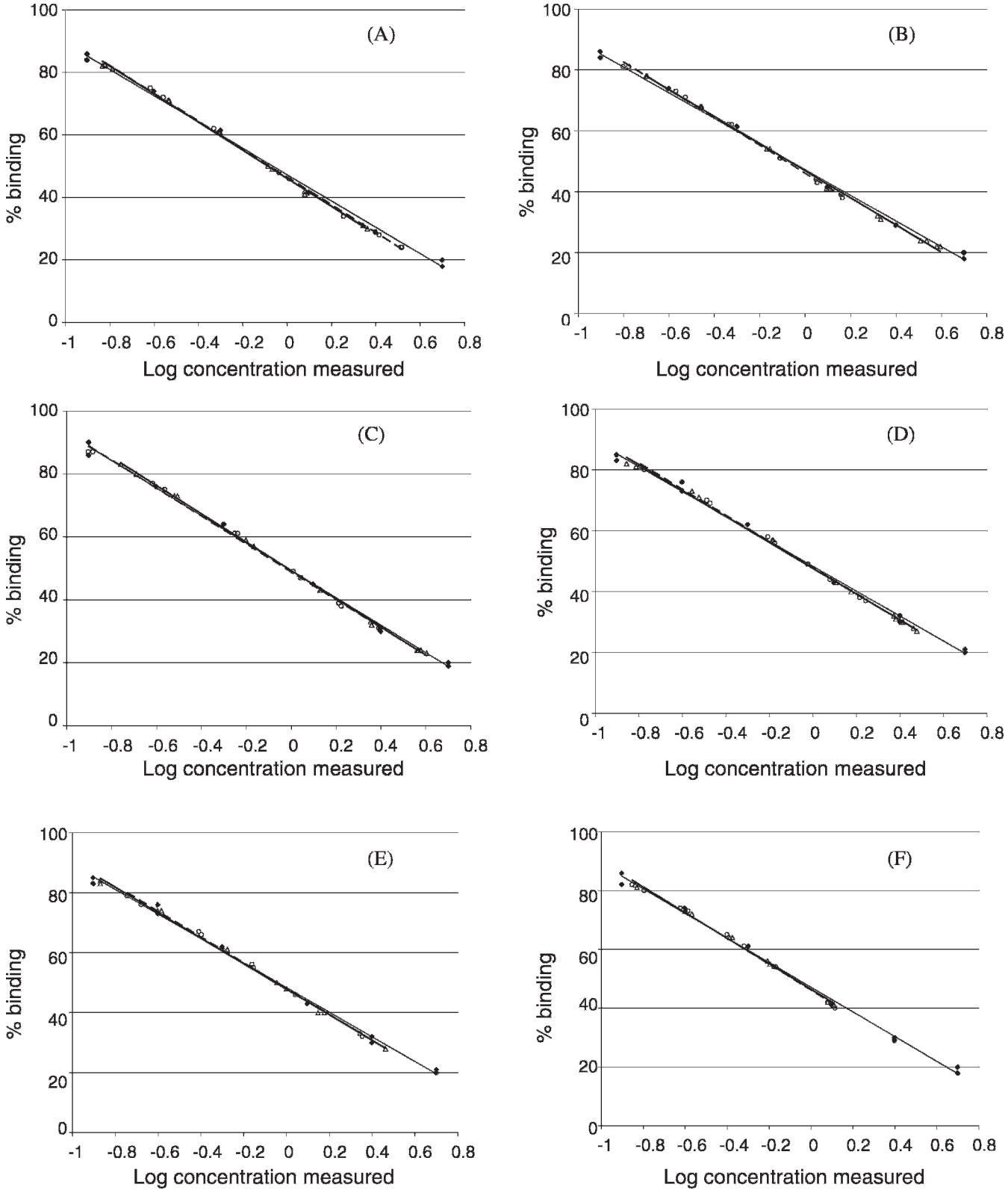

Figure 1. Parallelism of fecal glucocorticoid metabolite levels detected in feces using ^{125}I corticosterone radioimmunoassay (RIA) kits (MP Biomedicals, Solon, OH). We collected samples from May through August (dry season) and from December through February (wet season), during 2003–2005, in Tembe Elephant Park and Thanda Game Reserve, South Africa. For each species, we plotted the corticosterone standard curve (closed diamonds with thin solid trendline) and serial dilutions of herbivore fecal samples from the dry season (sample 1, open diamonds with dashed trendline) and wet season (sample 2, open triangles with thick solid trendline). Species included impala (A), kudu (B), nyala (C), giraffe (D), wildebeest (E), and zebra (F).

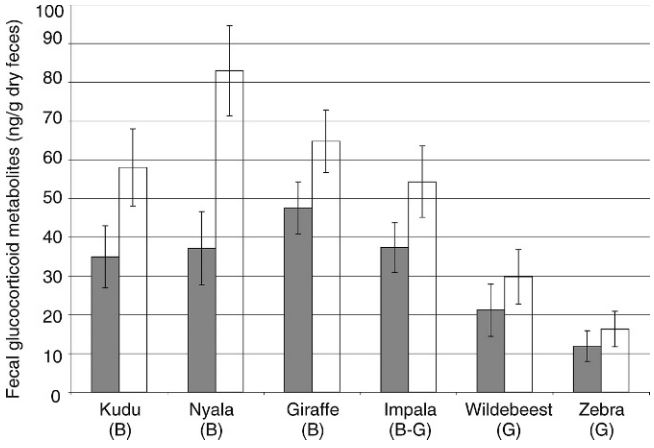

Figure 2. Biological validation of fecal glucocorticoid metabolite assays in 6 South African herbivores: effect of season and feeding guild. Mean concentration (with SE × 2) of fecal glucocorticoid metabolites (ng/g dry feces) for wet season (Dec–Feb, solid gray bar) and dry season (May–Aug, solid white bars) samples. We collected during 2003–2005 in Tembe Elephant Park and Thanda Game Reserve, South Africa. Browsers are indicated with (B) and grazers with (G); the impala is frequently a browser and grazer (B–G).

seasonal FGM production. Our study supported the findings of many other studies that have used this assay and shown FGM analysis to be an accurate means of quantifying the physiological stress in ungulate species (Wasser et al. 2000; Millspaugh et al. 2001, 2002). Fecal glucocorticoid measurements have been used to monitor adrenal activity in other free-ranging and captive African wildlife, including elephants (Foley et al. 2001, Ganswindt et al. 2003, Millspaugh et al. 2007), cheetah (*Acinonyx jubatus*; Jurke et al. 1997, Terio et al. 1998), white rhinoceros, and black rhinoceros (*Diceros bicornis*; Turner et al. 2002). To our knowledge, our study is the first to validate FGM assays for African Equidae species and Giraffidae. With knowledge that the RIA is appropriate for these species, studies directed at assessing effects of specific stressors are possible.

These 2 parks are typical of many similar parks throughout South Africa, in which management decisions are made based on multiple and often competing interests. Changing natural habitats, including human disturbances or species introductions, can elicit a physiological stress response in wildlife populations (Creel et al. 2002, Millspaugh et al. 2007, Thiel et al. 2008). One event can affect similar species differently (Palme et al. 2005); ecosystem changes that benefit some species may be detrimental to others. For example, introduction of large predators, such as lions, has been an issue in Tembe Elephant Park (Hunter et al. 2007). In Yellowstone National Park, Creel et al. (2007) noted lower progesterone value in elk as predation pressure increased, indicating that predation can affect some hormone levels in prey species. Our work, when combined with other research, supports the utility of noninvasive FGM measures as a means to forewarn the detrimental impacts of various stressors on the landscape (Creel et al. 2002). Compared with field observations, such as behavioral monitoring, collection of fecal material is straightforward, provided critical collection and storage protocols are followed (Millspaugh and Washburn 2004).

It is likely that differences in FGM levels we observed could be due to changing food abundance and selection. Seasonal differences in herbivore FGM levels have been demonstrated in white-tailed deer (*Odocoileus virginianus*; Millspaugh et al. 2002), elk (*Cervus elaphus*; Millspaugh et al. 2001), and Pyrenean chamois (*Rupicapra pyrenaica pyrenaica*; Dalmau et al. 2007). Distinct seasonal variation in FGM levels also has been documented in birds (Romero et al. 1997, Romero and Wingfield 1998) and rodents (Harper and Austad 2001). Although the mechanism for seasonal variation has yet to be determined, such variability could be due to changes in reproductive behavior, day length, and temperature (Harper and Austad 2001). In South Africa, seasonal rainfall has a profound impact on plant and animal communities by affecting the rate of plant growth and thus, available forage for herbivores.

The browser species we studied demonstrated a greater difference in FGM levels between seasons than did the grazer and browser–grazer species, but the reason for this variation is unknown. However, we cannot generalize that browsers live a more stressed existence nor can we make cross species comparisons. In herbivore species, the level of indigestible fiber in the diet directly affects fecal bulk and may have diluted the overall concentration of FGMs in a fecal sample (Goymann 2005). Longer gastrointestinal transit time allows for greater bacterial breakdown of FGMs and allows metabolites to be reabsorbed and reprocessed by the liver through enterohepatic circulation (Bokkenheuser and Winter 1980, Palme 2005). Certain dietary nutrients can affect FGM levels by binding hormone, changing gastrointestinal transit time or gut flora (Goymann 2005). Although we demonstrated that FGM analysis can detect seasonal variation in these species, we cannot determine the source of this variation, given the number of possibilities present. Of critical importance is acknowledging that this variation exists, which confirmed that we are able to detect biologically meaningful changes in FGM levels.

Although the RIA kit we used has been validated for a wide range of species (Wasser et al. 2000), clear differences occur in fecal composition, diet, and glucocorticoid metabolism among taxa that can influence the types of metabolites found in feces (Palme 2005). Because we are not measuring native corticosterone in the feces, it is essential to validate the assay methodology to ensure that the suite of metabolites being detected is truly indicative of the level of adrenal activity in the species being studied (Wasser et al. 2000, Touma and Palme 2005). The MP Biomedicals antibody has been extensively validated; it showed the highest level of cross-reactivity with major fecal metabolites of corticosterone in 11 of 12 species studied, compared with 3 other antibodies (Wasser et al. 2000). The RIA does not provide information about relative abundance of individual metabolites but instead provides the concentration of all metabolites in the fecal extract. Identification of individual metabolites would be possible with high-performance liquid chromatography (Goymann 2005, Palme 2005), but at this

time, we have not determined a biological significance associated with any individual metabolites. Thus, we believe it is most appropriate to monitor net metabolite concentration to detect effects of external stimuli and identify species differences in FGM levels.

MANAGEMENT IMPLICATIONS

We validated an RIA that is capable of accurate and reliable quantification of FGM levels in 6 South African herbivores. Combined with other validation studies, our research demonstrates the utility of this RIA for FGM quantification in a diversity of wildlife species. Managers can use our reported optimal dilutions for future work on these species and the FGM levels provide reference values that were previously not available. For South African wildlife managers, this validation represented the first necessary step to successfully monitor the physiological stress response of several important wildlife species. However, proper implementation and interpretation of FGM assays should consider normal seasonal rhythms and avoid cross-species comparisons.

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